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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(54) Title:</b> <b>A PROCESS FOR THE PRODUCTION OF TRANSGENIC PLANTS WITH INCREASED NUTRITIONAL VALUE VIA THE EXPRESSION OF MODIFIED 2S STORAGE ALBUMINS</b>  <b>(57) Abstract</b> <p>The invention pertains to a process for producing transgenic plants with increased nutritional value. It comprises: cultivating plants obtained from regenerated plant cells or from seeds of plants obtained from said regenerated plant cells over one or several generations, whose genetic patrimony, replicable with said plants, comprises a precursor-coding nucleic acid sequence encoding the precursor of a 2S albumin storage protein and placed under the control of a promoter capable of directing gene expression in plants, said precursor-coding nucleic acid being modified in a nonessential region of its relevant sequence which encodes the mature 2S albumin or a subunit thereof with a nucleic acid insert in appropriate reading frame relationship with the surrounding part of said relevant sequence, said insert including a determined segment encoding an heterologous determined polypeptide containing appropriate aminoacid such as lysine and/or methionine and/or threonine and/or phenylalanine and/or tryptophane and/or leucine and/or valine and/or isoleucine.</p>		

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**A process for the production of transgenic plants  
with increased nutritional value via the  
expression of modified 2S storage albumins**

5           This invention relates to a process for the production  
of plants with increased content of appropriate aminoacids  
having high nutritional properties through the modification  
of plant genes encoding plant storage proteins, more particu-  
larly the 2S albumins.

10           More particularly, the invention aims at providing  
genetically modified plant DNA and plant live material in-  
cluding said genetically modified DNA replicable with the  
cells of said plant material, which genetically modified  
plant DNA contains sequences encoding for a polypeptide  
15           containing said appropriate aminoacids which expression is  
under the control of a suitable plant promoter.

          A further object of the invention is to take advantage  
of the capacity of 2S albumins to be produced in large  
amounts in plants.

20           A further object of the invention is to take advantage  
of a hypervariable region of the 2S albumins, which supple-  
mentation with a number of said appropriate aminoacid codons  
in said hypervariable region of the gene encoding said 2S  
albumins, do not disturb the correct expression, processing  
25           and transport of said produced modified storage proteins in  
the protein bodies of the plants.

          Animals and men obtain directly or indirectly their  
essential aminoacids by eating plants. These essential  
aminoacids include lysine, thryptophane, threonine, methion-  
30           ine, phenylalanine, leucine, valine and isoleucine. For the  
easiness of the language these aminoacids are called "appro-  
priate aminoacids". Rather recently, agricultural scien-  
tists concerned with the world's hungry problem, concentrat-  
ed their work on developing plants with high nutritional  
35           yield. These new varieties, obtained through breeding in

the most cases, were richer in carbohydrates but usually poorer in essential proteins than the wild type varieties from which they were derived. Currently, increasing recognition of the role of plants in supplying essential aminoacids to the animal world had led to emphasis on the development of new food plants having a better aminoacid content. Classical breeding however has limitations for achieving this goal. Molecular genetics, on the contrary, offers a possibility to overcome these difficulties. Reference is made to the European patent application 80208418 and the communication of Brown et al., 1986, in which a gene encoding a corn seed storage protein, (the so called zeins) is modified by the addition of sequences encoding lysine codons.

Seed storage proteins represent up to 90% of total seed protein in seeds of many plants. They are used as a source of nutrition for young seedlings in the period immediately after germination. The genes encoding them are strictly regulated, being expressed in a highly tissue specific and stage specific fashion (Walling et al., 1986; Higgins, 1984). Thus they are expressed almost exclusively in developing seed, and different classes of seed storage proteins may be expressed at different stages in the development of the seed. They are generally restricted in their intercellular location, being stored in membrane bound organelles called protein bodies or protein storage vacuoles. These organelles provide a protease-free environment, and often also contain protease inhibitors. A related group of proteins, the vegetative storage proteins, have similar aminoacid compositions and are also stored in specialized vacuoles, but are found in leaves instead of in seeds (Staswick, 1988). These proteins are degraded upon flowering, and are thought to serve as a nutritive source for developing seeds.

The expression of foreign genes in plants is well established (De Blaere et al., 1987). In several cases seed storage protein genes have been transferred to other plants. In most of these cases it was shown that within its new environment the transferred seed storage protein gene is expressed in a tissue specific and developmentally regulated manner (Beachy et al., 1985; Sengupta-Gopalan et al., 1985; Marris et al., 1988; Ellis et al., 1988; Higgins et al., 1986, Okamura et al., 1986). It has also been shown in at least two cases that foreign seed storage proteins are located in the protein bodies of the host plant (Greenwood and Chrispeels, 1985; Hoffman et al., 1987). It has further been shown that stable and functional messenger RNA's can be obtained if a cDNA, rather than a complete gene including introns, is used as the basis for the chimeric gene (Chee et al., 1986).

Storage proteins are generally classified on the basis of solubility and size (more specifically sedimentation rate, for instance as defined by Svedberg (in Stryer, L., Biochemistry, 2nd ed., W.H. Freeman, New York, page 599)). A particular class of seed storage proteins has been studied, the 2S seed storage proteins, which are water soluble albumins. They represent a significant proportion of the seed storage proteins in many plants (Youle and Huang, 1981) (Table I) and their small size and consequently simpler structure makes them an attractive target for modification (see also patent application EP 87 402 348.4). Several 2S storage proteins have been characterized at either the protein, cDNA or genomic clone levels (Crouch et al., 1983; Sharief and Li, 1982; Ampe et al., 1986; Altenbach et al., 1987; Ericson et al., 1986; De Castro et al., 1987; Scofield and Crouch, 1987; Josefsson et al., 1987; EP 87.4023484, Krebbers et al., 1988). 2S albumins are formed in the cell from two subunits of 6-9 and 3-4 kilodaltons (kd) respectively, which are linked by disulfide bridges.

5 The work in the references above showed that 2S albumins are synthesized as complex prepropeptides whose organization is shared between the 2S albumins of many different species and are shown diagrammatically for three of these species in figure 1. Several complete sequences are shown in figure 2.

10 As to Fig. 2 relative to protein sequences of 2S albumins, the following observations are made. For *B. napus*, *B. excelsia*, and *A. thaliana* both the protein and DNA sequences have been determined, for *R. communis* only the protein sequence is available (*B. napus* from Crouch et al., 1983 and Ericson et al., 1986; *B. excelsia* from Ampe et al., 1986, De Castro et al., 1987 and Altenbach et al., 1987, *R. communis* from Sharief and Li, 1982). Boxes indicate homologies, and raised dots the position of the cysteines.

15 Comparison of the protein sequences at the beginning of the precursor with standard consensus sequences for signal peptides reveals that the precursor has not one but two segments at the amino terminus which are not present in the mature protein, the first of which is a signal sequence  
20 (Perlman and Halvorson, 1983) and the second of which has been designated as the amino terminal processed fragment (the so-called ATPF). Signal sequences serve to ensure the co-translational transport of the nascent polypeptide across the membrane of the endoplasmic reticulum (Blobel, 1980), and are  
25 found in many types of proteins, including all seed storage proteins examined to date (Herman et al., 1986). This is crucial for the appropriate compartmentalization of the protein. The protein is further folded in such a way that correct disulfide bridges are formed. This process is probably  
30 localized at the luminal site of the endoplasmic reticulum membrane, where the enzyme disulfide isomerase is localized (Roden et al., 1982; Bergman and Kuehl, 1979). After translocation across the endoplasmic reticulum membrane it is  
35 thought that most storage proteins are transported via said

endoplasmic reticulum to the Golgi bodies, and from the latter in small membrane bound vesicles ("dense vesicles") to the protein bodies (Chrispeels, 1983; Craig and Goodchild, 1984; Lord, 1985). That the signal peptide is removed co-translationally implies that the signals directing the further transport of seed storage proteins to the protein bodies must reside in the remainder of the protein sequence present. Zeins and perhaps some other prolamins deviate from this pathway; indeed the protein bodies are formed by budding directly off of the endoplasmic reticulum (Larkins and Hurkman, 1978). As already of record, 2S albumins contain sequences at the amino end of the precursor other than the signal sequence which are not present in the mature polypeptide. This is not general to all storage proteins. This amino terminal processed fragment is labeled ATPF in figure 1.

In addition, as shown in figure 1, several aminoacids located between the small and large subunits in the precursor are removed (labeled IPF in the figure, which stands for internal processed fragment). Furthermore, several residues are removed from the carboxyl end of the precursor (labeled CTPF in the figure which stands for carboxyl terminal processed fragment). The cellular location of these latter processing steps is uncertain, but is most likely the protein bodies (Chrispeels et al., 1983; Lord, 1985). As a result of these processing steps the small subunit and the large subunit remain. These are linked by disulfide bridges, as discussed below.

When the protein sequences of 2S albumins of different plants are compared strong structural similarities are observed. This is more particularly illustrated by figure 2 which provides the aminoacid sequences of the small subunit and large subunit respectively of representative 2S storage seed albumin proteins of different plants, i.e.,:

R. comm. : Ricinus communis

A. thali.: Arabidopsis thaliana

B. napus : Brassica napus

B. excel.: Bertholletia excelsia (Brazil nut)

It must be noted that in Fig. 2:

- 5       -     the aminoacid sequences of said subunits extend on  
several lines; the cysteine groups of the aminoacid  
sequences of the exemplified storage proteins and identical  
aminoacids in several of said proteins have been  
brought into vertical alignment; the hyphen signs which  
10       appear in some of these sequences represent absent  
aminoacids, in other words direct linkages between the  
closest aminoacids which surrounded them;
- the aminoacid sequences which in the different proteins  
are conserved are framed.

15       It will be observed that all the sequences contain  
eight cysteine residues (the first and second in the small  
subunit, the remainder in the large subunit) which could  
participate in disulfide bridges as diagrammatically shown  
in Fig. 3, which represents a hypothetical model (for the  
20       purpose of the present discussion) rather than a representa-  
tion of the true structure of the 2S albumin of Arabidopsis  
thaliana.

      Said hypothetical model has been inspired by the dis-  
ulfide bridge mediated loop-formation of animal albumins,  
25       such as serum albumins (Brown, 1976), alpha-fetoprotein  
(Jagodzinski et al., 1987; Morinaga et al., 1983) and the  
vitamine D binding protein where analogous constant C-C  
doublets and C-X-C triplets were observed (Yang et al.,  
1985).

30       As can be seen on Fig. 2, the regions which are interca-  
lated between the first and second cysteines, between the  
fifth and sixth cysteines, and between the seventh and eight  
cysteines of the mature protein show a substantial degree of  
conservation or similarity. It would thus seem that these  
35       regions are in some way essential for the proper folding

and/or stability of the protein when synthesized in the plants. An exception to this conservation consist in the distance between the sixth and seventh

5 cysteine residues. This suggests that these arrangements are structurally important, but that some variation is permissible in the large subunit between said sixth and seventh cysteines where little conservation of aminoacids is observed. An analogous suggestion has been made by Slightom and Chee  
10 (1987), where the viciline type seed storage proteins from peas were compared. These authors indeed suggest that aminoacid replacement mutations designed to increase the number of sulphur containing aminoacids should be placed in regions which show little or no conservation of aminoacid sequences. The authors however conclude that the proof that such modifications  
15 can be tolerated will need to be tested in the seeds of transgenic plants. Moreover, the teaching provided in their paper on the properties of the through deletion modified storage protein concerns only the influence on expression levels and not on processing of said storage proteins.  
20

An embodiment of this invention is the demonstration that a well chosen region of the 2S albumin allows variation without altering the properties and correct processing of said modified storage protein in plant cells of transgenic plants.  
25

This region (diagrammatically shown in Fig. 3 by an enlarged hatched portion) will in the examples hereafter referred to be termed as the "hypervariable region". Fig. 3 also shows the respective positions of the other parts of the precursor sequence, including the "IPF" section separating  
30 the small subunit and large subunit of the precursor, as well as the number of aminoacids (aa) in substantially conserved portions of the protein subunits cysteine residues. The processing cleavage sites (as determined by Krebbers et al.,  
35 1988) are shown by symbols.

The seeds of many plants contain albumins of approximately the same size as the storage proteins discussed above. However, for ease of language, this document will use the term "2S albumins" to refer to seed proteins whose genes encode a peptide precursor with the general organization shown in figure 1 and which are processed to a final form consisting of two subunits linked by disulfide bridges. The process of the invention for producing plants with an increased content of appropriate aminoacids comprises :

cultivating plants obtained from regenerated plant cells or from seeds of plants obtained from said regenerated plant cells over one or several generations, wherein the genetic patrimony or information of said plant cells, replicable within said plants, includes a nucleic acid sequence, placed under the control of a plant promoter, which can be transcribed into the mRNA encoding at least part of the precursor of a 2S albumin including the signal peptide of said plant, said nucleic acid being hereafter referred to as the "precursor encoding nucleic acid"

. wherein said nucleic acid contains a nucleotide sequence (hereafter termed the "relevant sequence") which relevant sequence comprises a nonessential region modified by a heterologous nucleic acid insert forming an open reading frame in reading phase with the non modified parts surrounding said insert in said relevant sequence.

. wherein said insert includes a nucleotide segment encoding a polypeptide containing appropriate aminoacids.

It will be appreciated that under the above mentioned conditions each and every cell of the cultivated plant will include the modified nucleic acid. Yet the above defined recombinant or hybrid sequence will be expressed at high levels constitutively or only or mostly in certain organs of

the cultivated plants dependent on which plant promoter has been chosen to conduct its expression. In the case of seed-specific promoters the hybrid storage protein will be produced mostly in the seeds.

5 It will be understood that the "heterologous nucleic acid insert" defined above consists of an insert which contains nucleotide sequences which at least in part, may be foreign to the natural nucleic acid encoding the precursor of the 2S albumins of the plant cells concerned and encode the appropriate aminoacids. Most generally the segment encoding polypeptide containing said appropriate aminoacids will itself be foreign to the natural nucleic acid encoding the precursor of said storage protein. Nonetheless, the term "heterologous nucleic acid insert" does also extend to an insert containing a segment as above-defined normally present in the genetic patrimony or information of said plant cells, the "heterologous" character of said insert then addressing to the different genetic environment which surrounds said insert.

20 In the preceding definition of the process according to the invention the so-called "nonessential region" of the relevant sequence of said nucleic acid encoding the precursor, consists of a region whose nucleotide sequence can be modified either by insertion into it of the above defined insert or by replacement of at least part of said nonessential region by said insert, yet without disturbing the stability and correct processing of said hybrid storage protein as well as its transport into the above-said protein bodies. Sequences consisting of said insert or replacement and representing the coding region for a polypeptide containing appropriate aminoacids can either be put in as synthetic oligomers or as restriction fragments isolated from other genes, as thought by Brown, 1986. The total length of the hybrid storage protein may be longer or shorter than the total length of the non-modified 2S albumin.

With respect to the choice of the region to be modified, the present invention is clearly distinguishable from other work which has been done in this field. Reference is made to DD-A-240911 patent from the Akademie der Wissenschaften der DDR where legumin genes from Vicia faba, (glutine and prolamine) were modified in vitro with sequences encoding methionine. As place of insert a natural occurring PstI site has been chosen. At the EMBO workshop "Plant storage protein genes", (Breisach, FRG, September 1986) the authors presented their work and informed the audience that plant transformation experiments were just started with the modified gene. No further results have yet been published.

Reference is also made to patent application WO-A-87/07299 and corresponding publication of Radke et al., 1988. These papers describe the modification of the napin gene, which encodes the 2S albumin of Brassica napus, by a nucleotide sequence encoding nine aminoacid residues including 5 consecutive methionines. The region of modification is a naturally occurring SstI site within the region encoding the mature protein. Such a modification would result in a insertion directly adjacent to a cysteine residue and moreover in a region between two cysteines, namely the 4th and the 5th cysteines of the mature protein which correspond with the 2nd and 3rd cysteines of the large subunit, whose length is strongly conserved (see above). We believe such a modification is likely to disrupt a normal folding and stability of the 2S albumin (see also EP 87 402 348.4). Moreover, above cited references provide no evidence that the desired modified 2S albumin was successfully synthesized, correctly processed or correctly targeted.

In the present invention the precursor-coding nucleic acid referred to above may of course originate from the same plant species as that which is cultivated for the purpose of the invention. It may however originate from another plant species, in line with the teachings of Beachey et al., 1985 and Okamuro et al., 1986 already of record.

In a similar manner the plant promoter may originate from the same plant species or from another, subject in the last instance to the capability of the host plant's polymerases to recognize it. It may act constitutively or in a tissue-specific manner, such as, but not limited to, seed-specific promoters.

Regions such as the ones at the end of the small subunit, at the beginning or end of the large subunit, show differences of such a magnitude that they can be held as presumably having no substantial impact on the final properties of the protein. The extreme carboxyl terminus of the small subunits and the amino terminus of the large subunit may, however, be involved in the processing of the internal processed fragment. A region which does not seem essential, consists of the middle position of the region located in the large subunit, between the sixth and the seventh cysteine of the nature protein, but not immediately adjacent and at least 3 aminoacids separated from said cysteines. Thus in addition to the absence of similarity at the level of the aminoacid residues, there appears a difference in length which makes that region eligible for substitutions in the longest 2S albumins and for addition of aminoacids in the shortest 2S albumins or for elongation of both. The same should be applicable at approximately of the end of the first third part of the same region between said sixth and seventh cysteine; see the sequence of *R. communis* which is much shorter at that region than the corresponding regions of the other exemplified 2S proteins.

It is of course realized that caution must be exercised against hypotheses based on arbitrary choices as concerns the bringing into line of similar parts of proteins which elsewhere exhibit substantial differences. Nevertheless such comparisons have proven in other domains of genetics to provide the man skilled in the art with appropriate guidance to reasonably infer from local structural differences, on the

one hand, and from local similarities, on the other hand, in similar proteins of different sources, which parts of such proteins can be modified and which parts cannot, when it is sought to preserve some basic properties of the non modified protein in the same protein yet locally modified by a foreign or heterologous sequence.

The choice of the adequate nonessential regions to be used in the process of the invention will also depend on the length of the polypeptide containing the appropriate aminoacids. Basically the method of the invention allows the modification of said 2S albumins by the insertion and/or partial substitution into the precursor nucleic acid of sequences encoding up to 100 aminoacids.

When the complete protein sequence of the region to be inserted into a 2S albumin has been determined, the nucleotide sequence to encode said protein sequence must be determined. It will be recognized that while perhaps not absolutely necessary the codon usage of the encoding nucleic acid should where possible be similar to that of the gene being modified.

The person skilled in the art will have access to appropriate computer analysis tools to determine said codon usage.

Any appropriate genetic engineering technique may be used for substituting the insert for part of the selected precursor-coding nucleic acid or for inserting it in the appropriate region of said precursor-coding nucleic acid. The general in vitro recombination techniques followed by cloning in bacteria can be used for making the chimeric genes. Site-directed mutagenesis can be used for the same purposes as further exemplified hereafter. DNA recombinants, e.g. plasmids suitable for the transformation of plant cells can also be produced according to techniques disclosed in current technical literature. The same applies finally to the production of transformed plant cells in which the hybrid storage protein encoded by the relevant parts of the selected

precursor-coding nucleic acid can be expressed. By way of example, reference can be made to the published European applications no. 116 718 or to International application WO 84/02913 and, which disclose appropriate techniques to that effect.

When designing the sequences rich in appropriate aminoacids, care must be taken that the resulting peptide containing said appropriate aminoacids does not influence the stability of the modified 2S albumin. Certain insertions may indeed disrupt the structure of the protein. For example, long stretches of methionines may result in rod shaped helices which would result in instabilities due to disruption of normal folding patterns. Thus such sequences must occasionally include aminoacids which interrupt the helical structure.

The procedures which have been disclosed hereabove apply to the adequate modification of the nonessential region of any of 2S albumins by an heterologous insert containing a DNA sequence encoding a peptide containing appropriate aminoacids with nutritional properties and then to the transformation of the relevant plants with the chimeric gene obtained for the production of a hybrid protein containing the sequence of said peptide in the cells of the relevant plant. Needless to say that the person skilled in the art will in all instances be able of selecting which of the existing techniques would at best fulfill its needs at the level of each step of the production of such modified plants, to achieve the best production yields of said hybrid storage protein.

For instance the following process can be used in order to exploit the capacity of a 2S albumin, to be used as a suitable vector for the production of plants with increased nutritional value, by inserting in said 2S albumins nucleotide codons encoding methionine and/or lysine and/or thryptophane and/or threonine and/or phenylalanine and/or leucine and/or valine and/or isoleucine when the corresponding

precursor-coding nucleic acid has been sequenced. Such process then comprises:

5 1) locating and selecting one of said relevant sequences of the precursor-coding nucleic acid which comprises a nonessential region encoding a peptide sequence which can be modified by substituting an insert for part of it or by inserting of said insert into it, which modification is compatible with the conservation of the configuration of said 2S albumins and this preferable by determining  
10 the relative positions of the codons which encode the successive cysteine residues in the mature protein or protein subunits of said 2S albumins and identifying the corresponding successive nucleic acid regions located upstream of, between, and downstream of said codons with-  
15 in said sub-sequences of the precursor-coding nucleic acid and identifying in said successive regions those parts which undergo variability in either aminoacid sequence or length or both from one plant species to another as compared with those other regions which do exhibit  
20 substantial conservation of aminoacid sequence in said several plant species, one of said nucleotide regions being then selected for the insertion therein of the nucleic acid insert as described hereunder.

25 An alternative would consist of studying any 3-D structures which may become available in the future.

30 2) inserting a nucleic acid insert in the selected region of said precursor nucleic acid in appropriate reading frame relationship with the non-modified parts of said relevant sequence, which insert includes a determined segment encoding a peptide containing all or part of the above mentioned appropriate aminoacids.

35 3) inserting the modified precursor-coding nucleic acid obtained in a plasmid suitable for the transformation of plant cells which can be regenerated into full

seed-forming plants, wherein said insertion is brought under the control of regulation elements, particularly a plant promoter capable of providing for the expression of the open reading-frames associated therewith in said plants;

- 4) transforming a culture of such plant cells with such modified plasmid;
- 5) assaying the expression of the chimeric gene encoding the hybrid storage protein and, when achieved;
- 6) regenerating said plants from the transformed plant cells obtained and growing said plants up to maturity.

In the case the chimeric gene is under the control of a seed specific promotor, growing up the transformed plants to seeds must precede step 5)

Hence embodiment as described under 1) of the invention hereabove provides that in having the hybrid 2S albumins in a plant, it will pass the plant protein disulfide isomerase during membrane translocation, thus increasing the chances that the correct disulfide bridges be formed in the hybrid precursor as in its normal precursor situation, on the one hand

The invention further relates to the recombinant nucleic acids themselves for use in the process of the invention; particularly to the

- recombinant precursor encoding nucleic acid defined in the context of said process;
- recombinant nucleic acids containing said modified precursor encoding nucleic acid under the control of a plant promoter, whether the latter originates from the same DNA as that of said precursor coding nucleic acid or from another DNA of the same plant from which the precursor encoding nucleic acid is derived, or from a DNA of another plant, or from a non-plant organism provided that it is capable of directing gene expression in plants.

- vectors, more particularly plant plasmids e.g., Ti-derived plasmids modified by any of the preceding recombinant nucleic acids for use in the transformation of the above plant cells.

5 The invention also relates to the regenerable source of the hybrid 2S albumin, which is formed of in the cells of a seed-forming-plant, which plant cells are capable of being regenerated into the full plant or seeds of said seed-forming plants wherein said plants or seeds have been obtained as a result of one or several generations of the plants resulting from the regeneration of said plant cells, wherein further the DNA supporting the genetic information of said plant cells or seeds comprises a nucleic acid or part thereof, including the sequences encoding the signal peptide, which can be transcribed in the mRNA corresponding to the precursor of a 2S albumin of said plant, placed under the control of a plant specific promoter, and

10 . wherein said nucleic acid sequence contains a relevant modified sequence encoding the mature 2S storage protein or one of the several sub-sequences encoding for the corresponding one or several sub-units of said mature 2S albumins,

15 . wherein further the modification of said relevant sequence takes place in one of its nonessential regions and consists of a heterologous nucleic acid insert forming an open-reading frame in reading phase with non modified parts which surround said insert in the relevant sequence,

20 . wherein said insert consists of a nucleotide segment encoding a peptide containing methionine and/or lysine and/or thryptophane and/or threonine and/or phenylalanine, and/or leucine and/or valine and/or isoleucine.

25 It is to be considered that although the invention should not be deemed as being limited thereto, the nucleic inserts encoding the above mentioned appropriate aminoacids

will in most instances be man-made synthetic oligonucleotides or oligonucleotides derived from procaryotic or eucaryotic genes or of from cDNAs derived of procaryotic or eucaryotic RNAs, all of which shall normally escape any possibility of being inserted at the appropriate places of the plant cells or seeds of this invention through biological processes, whatever the nature thereof. In other words, these inserts are "non plant variety specific", specially in that they can

be inserted in different kinds of plants which are genetically totally unrelated and thus incapable of exchanging any genetic material by standard biological processes, including natural hybridization processes.

Thus the invention further relates to the seed forming plants themselves which have been obtained from said transformed plant cells or seeds, which plants are characterized in that they carry said hybrid precursor-coding nucleic acids associated with a plant promoter in their cells, said inserts however being expressed and the corresponding hybrid protein produced in the cells of said plants.

There follows an outline of a preferred method which can be used for the modification of a 2S albumin gene and its expression in the seeds obtained from the transgenic plants. The outline of the method given here is followed by a specific example. It will be understood from the person skilled in the art that the method can be suitably adapted for the modification of other 2S albumin genes.

1. Replacement or supplementation of the hypervariable region of the 2S albumin gene by a sequence encoding peptide containing appropriate aminoacids which possess nutritional properties.

Either the cDNA or the genomic clone of the 2S albumin can be used. Comparison of the sequences of the hypervariable regions of the genes in figure 2 shows that they vary in length. Therefore if the sequence encoding a peptide contain-

ing the appropriate aminoacids is short and a 2S albumin with a relatively short hypervariable region is used, said sequence of interest can be inserted. Otherwise part of the hypervariable region is removed, to be replaced by the insert containing a larger segment or sequence encoding the peptide containing the appropriate aminoacids. In either case the modified hybrid 2S albumin may be longer than the native one. In either case two standard techniques can be applied; convenient restriction sites can be exploited, or mutagenesis vectors (e.g. Stanssens et al. 1987) can be used. In both cases, care must be taken to maintain the reading frame of the message.

The sequence encoding the signal peptide of the precursor of the storage protein used either belongs to this precursor or can be a substitute sequence coding for the signal peptide or peptides of an heterologous storage protein.

2. The altered 2S albumin coding region is placed under the control of a plant promoter. Preferred promoters include the strong constitutive exogeneous plant promoters such as the promoter from cauliflower mosaic virus directing the 35S transcript (Odell, J.T. et al., 1985), also called the 35S promoter; the 35S promoter from the CAMV isolate Cabb-JI (Hull and Howell, 1987), also called the 35S3 promoter; the bidirectional TR promoter which drives the expression of both the 1' and the 2' genes of the T-DNA (Velten et al., 1984).

Alternatively a promoter can be utilized which is not constitutive but specific for one or more tissues or organs of the plant. Given by way of example such kind promoters may be the light inducible promoter of the ribulose-1, 5-bi-phosphate carboxylase small subunit gene (US patent application 821, 582), if the expression is desired in tissue with photosynthetic activity, or may be seed specific promoters.

A seed specific promoter is used in order to ensure subsequent expression in the seeds only. This may be of particular use, since seeds constitute an important food or feed source. Moreover, this specific expression avoids possible stresses on other parts of the plant. In principle the promoter of the modified 2S albumin can be used. But this is not necessary. Any other promoter serving the same purpose can be used. The promoter may be chosen according to its level of efficiency in the plant species to be transformed. In the examples below the 2S albumin promoter from the 2S albumin gene from Arabidopsis is used, which constitutes the natural promoter of the 2S albumin gene which is modified in said examples. Needless to say that other seed specific promoters may be used, such as the conglycinine promoter from soybean. If a chimeric gene is so constructed, a signal peptide encoding region must also be included, either from the modified gene or from the gene whose promoter is being used. The actual construction of the chimeric gene is done using standard molecular biological techniques described in Maniatis et al., 1982. (see example).

3. The chimeric gene construction is transferred into the appropriate host plant.

When the chimeric or modified gene construction is complete it is transferred in its entirety to a plant transformation vector. A wide variety of these, based on disarmed (non-oncogenic) Ti-plasmids derived from Agrobacterium tumefaciens, are available, both of the binary and cointegration forms (De Blaere et al., 1987). A vector including a selectable marker for transformation, usually antibiotic resistance, should be chosen. Similarly, the methods of plant transformation are also numerous, and are fitted to the individual plant. Most are based on either protoplast transformation (Marton et al., 1979) or formation of a small piece of tissue from the adult plant (Horsch et al., 1985). In the example below, the vector is a binary disarmed

Ti-plasmid vector, the marker is kanamycin resistance, and the leaf disc method of transformation is used.

5        Calli from the transformation procedure are selected on the basis of the selectable marker and regenerated to adult plants by appropriate hormone induction. This again varies with the plant species being used. Regenerated plants are then used to set up a stable line from which seeds can be harvested.

10       Further characteristics of the invention will appear in the course of the non-limiting disclosure of specific examples, particularly on the basis of the drawings in which:

- 15       - Figs. 1, 2 and 3 refer to overall features of 2S-albumins as already discussed above. The numbers refer to the number of aminoacids observed in the different fragments of the protein precursor.
- 20       - Fig. 4 represents the sequence of 1kb fragment containing the Arabidopsis thaliana 2S albumin gene and shows related elements. The NdeI site is underlined.
- 25       - Fig. 5 provides the protein sequence of the large subunit of the above Arabidopsis 2S protein together with related oligonucleotide sequences.
- 30       - Fig. 6A shows diagrammatically the successive phases of the construction of a chimeric 2S albumin Arabidopsis thaliana gene including the deletion of practically all parts of the hypervariable region and its replacement by a AccI site, the insertion of DNA sequences rich in methionine codons, given by way of of example in the following disclosure, in the AccI site, particularly through site-directed mutagenesis and the cloning of said chimeric gene in plant vector suitable for plant transformation.
- 35       - Fig. 6B shows diagrammatically the protein sequence of the large subunit of several Arabidopsis 2S albumins and indicates the region removed from the genes

encoding said 2S albumins, and shows diagrammatically where an AccI site has been created and how oligonucleotides rich in methionine codons are inserted into said AccI site in such a way that the open reading frame is maintained.

5

- Fig 7 diagrammatically compares the protein sequences of the large subunits of the unmodified 2S albumin, in which most of the hypervariable region has been deleted, and those of the modified 2S albumins. The resulting number of methionine residues are indicated.
- Fig. 8 shows the restriction sites and genetic map of a plasmid suitable for the performance of the above site-directed mutagenesis.
- Fig. 9 shows diagrammatically the different steps of the site-directed mutagenesis procedure of Stanessens et al (1987) as generally applicable to the modification of nucleic acid at appropriate places.
- Fig. 10 gives the restriction map of pGSC1703A.

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#### Example I :

As a first example of the method described, a procedure is given for the production of transgenic plant seeds with increased nutritional value by having inserted into their genome a modified 2S albumin protein from Arabidopsis thaliana having deleted its hypervariable region and replaced by way of example by a methionine rich peptide having 7 aminoacids with the following sequence : I M M M M R M. A synthetic oligomer encoding said peptide is substituted for essentially the entire part of the hypervariable region in a genomic clone encoding the 2S albumin of Arabidopsis thaliana. Only a few aminoacids adjacent to the sixth and seventh cysteine residues remained. This chimeric gene is under the control of its natural promoter and

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30

35

signal peptide. The process and constructions are diagrammatically illustrated in Fig. 6A, 6B and 7. The entire construct is transferred to tobacco, Arabidopsis thaliana and Brassica napus plants using an Agrobacterium mediated transformation system. Brassica napus is of particular interest, since this crop is widely used as protein source for animal feed.

Plants are regenerated, and after flowering the seeds are collected and the methionine content compared with untransformed plants.

#### 1. Cloning of the Arabidopsis thaliana 2S albumin gene.

The Arabidopsis thaliana gene has been cloned according to what is described in Krebbers et al., 1988. The plasmid containing said gene is called pAT2S1. The sequence of the region containing the gene, which is called AT2S1, is shown in figure 4.

#### 2. Deletion of the hypervariable region of AT2S1 gene and replacement by an AccI site.

Part of the hypervariable region of AT2S1 is replaced by the following oligonucleotide:

5'- CCA ACC TTG AAA GGT ATA CAC TTG CCC AAC - 3'  
30-mer

P T L K G I H L P N

in which the underlined sequences represent the AccI site and the surrounding ones sequences complementary to the coding sequence of the hypervariable region of the Arabidopsis 2S albumin gene to be retained. This results finally in the aminoacid sequence indicated under the oligonucleotide.

The deletion and substitution of part of the sequence encoding the hypervariable region of AT2S1 is done using site directed mutagenesis with the oligonucleotide as primer. The system of Stanssens et al. (1987) is used.

5 The Stanssens et al. method is described in EP 87 402 384.4. It makes use of plasmid pMac5-8 whose restriction and genetic map and the positions of the relevant genetic loci are shown in Fig. 8. The arrows denote their functional orientation.

10 fdT: central transcription terminator of phage fd; F1-ORI: origin of replication of filamentous phage f1; ORI: ColE1-type origin of replication; BLA/Ap<sup>R</sup> : region coding for B-lactamase; CAT/Cm<sup>R</sup> : region coding for chloramphenicol acetyl transferase. The positions of the amber mutations present in pMc5-8 (the bla-am gene does not contain the ScaI site) and

15 pMc5-8 (cat-am; the mutation eliminates the unique PvuII site) are indicated. Suppression of the cat amber mutation in both supE and supF hosts results in resistance to at least 25 ug/ml Cm. pMc5-8 confers resistance to  $\pm 20$  ug/ml and 100 ug/ml Ap upon amber-suppression in supE and supF strains respectively. The EcoRI, BclI and NcoI sites present in the wild-type cat gene (indicated with an asterisk) have been removed using mutagenesis techniques.

25 Essentially the mutagenesis round used for the above mentioned substitution is ran as follows. Reference is made to Fig. 9, in which the amber mutations in the Ap and Cm selectable markers are shown by closed circles. The symbol ^ represents the mutagenic oligonucleotide. The mutation itself is indicated by an arrowhead.

30 The individual steps of the process are as follows:

- Cloning of the HindIII fragment of pAT2S1 containing the coding region of the AT2S1 gene into pMa5-8 (I). This vector carries on amber mutation in the Cm<sup>R</sup> gene and

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specifies resistance to ampicillin. The resulting plasmid is designated pMacAT2S1 (see figure 6A step 1).

- Preparation of single stranded DNA of this recombinant (II) from pseudoviral particles.
- 5 - Preparation of a HindIII restriction fragment from the complementary pMc type plasmid (III). pMc-type vectors contain the wild type  $Cm^R$  gene while an amber mutation is incorporated in the Ap resistance marker.
- 10 - Construction of gap duplex DNA (hereinafter called gdDNA) gdDNA (IV) by in vitro DNA/DNA hybridization. In the gdDNA the target sequences are exposed as single stranded DNA. Preparative purification of the gdDNA from the other components of the hybridization mixture is not necessary.
- 15 - Annealing of the 30-mer synthetic oligonucleotide to the gdDNA (V).
- Filling in the remaining single stranded gaps and sealing of the nicks by a simultaneous in vitro Klenow DNA polymerase I / DNA ligase reaction (VI).
- 20 - Transformation of a mutS host, i.e., , a strain deficient in mismatch repair, selecting for  $Cm$  resistance. This results in production of a mixed plasmid progeny (VII).
- Elimination of progeny deriving from the template strand (pMa-type) by retransformation of a host unable to suppress amber mutations (VIII). Selection for  $Cm$  resistance results in enrichment of the progeny derived from the gapped strand, i.e., , the strand into which the mutagenic oligonucleotide has been incorporated.
- 25 - Screening of the clones resulting from the retransformation for the presence of the desired mutation. The resulting plasmid containing the deleted hypervariable region of AT2S1 is called pMacAT2S1C40 (see figure 6A step 2).
- 30

3. Insertion of sequences rich in methionine codons into the AT2S1 gene whose sequences encoding the hypervariable region have been deleted.

As stated above when the sequences encoding most of the hypervariable loop were removed an AccI site was inserted in its place. The sequences of interest will be inserted into this AccI site, but a second AccI site is also present in the HindIII fragment containing the modified gene. Therefore the NdeI-HindIII fragment containing the modified gene is subcloned into the cloning vector pBR322 (Bolivar, 1977) also cut with NdeI and HindIII. The position of the NdeI site in the 2S albumin gene is indicated in figure 4. The resulting subclone is designated pBRAT2S1 (Figure 6A, step 3).

In principle any insert desired can be inserted into the AccI site in pBRAT2S1. In the present example said insert encodes the following sequence: I.M.M.M.M.R.M. Therefore complementary oligonucleotides encoding said peptide are synthesized taking into account the codon usage of AT2S1 and ensuring the the ends of the two complementary oligonucleotides are complementary to the staggered ends of the AccI site, as shown here (the oligonucleotides are shown in bold type) :

5' GT ATA ATG ATG ATG ATG CGC ATG ATAC 3'  
3' CA TAT TAC TAC TAC TAC GCG TAC TATG 5'

The details of this insertion, showing how the reading frame is maintained, are shown in figure 6B. The two oligonucleotides are annealed and ligated with pBRAT2S1 digested with AccI (figure 6A, step 4). The resulting plasmid is designated pAD4.

#### 4. Reconstruction of the complete modified AT2S1 gene with its natural promoter.

The complete chimeric gene is reconstructed as follows (see figure 6A): The clone pAT2S1Bg contains a 3.6kb BglII fragment inserted in the cloning vector pJB65 (Botterman et al., 1987) which encompasses not only the 1.0kb HindIII fragment containing the coding region of the gene AT2S1 but sufficient sequences upstream and downstream of this fragment to contain all necessary regulatory elements for the proper expression of the gene. This plasmid is cut with HindIII and the 5.2kb fragment (i.e., that portion of the plasmid not containing the coding region of AT2S1) is isolated. The clone pAT2S1 is cut with HindIII and NdeI and the resulting 320 bp HindIII-NdeI fragment is isolated. This fragment represents the one removed from the modified 2S albumin in the construction of pBRAT2S1 (step 3 of figure 6A) in order to allow the insertion of the oligonucleotides in step 4 of figure 6A to proceed without the complications of an extra AccI site. These two isolated fragments are then ligated in a three way ligation with the NdeI-HindIII fragment from pAD4 (figure 6A, step 5) containing the modified coding sequence. Individual transformants can be screened to check for appropriate orientation of the reconstructed HindIII fragment within the BglII fragment using any of a number of sites. The resulting plasmid, pAD17, consists of a 2S albumin gene modified only in the hypervariable region, surrounded by the same flanking sequences and thus the same promoter as the unmodified gene, the entirety contained on a BglII fragment.

#### 5. Transformation of plants.

The BglII fragment containing the chimeric gene is inserted into the BglII site of the binary vector pGSC1703A (Fig. 10) (see also Fig. 6A step 6). The resultant plasmid is designated pTAD12. Vector pGSC1703A contains functions

for selection and stability in both E. coli and A. tumefaciens, as well as a T-DNA fragment for the transfer of foreign DNA into plant genomes (Deblaere et al., 1987). It further contains the bi-directional TR promotor (Velten et al., 1984) with the neomycin phosphotransferase protein coding region (neo) and the 3' end of the ocs gene on one side, and a hygromycin transferase gene on the other side, so that transformed plants are both kanamycin and hygromycin resistant. This plasmid does not carry an ampicillin resistance gene, so that carbenicillin as well as claforan can be used to kill Agrobacterium after the infection step. Using standard procedures (Deblaere et al., 1987), pTAD12 is transferred to the Agrobacterium strain C58C1Rif carrying the plasmid pMP90 (Koncz and Schell, 1986). The latter provides in trans the vir gene functions required for successful transfer of the T-DNA region to the plant genome. This Agrobacterium is then used to transform plants. Tobacco plants of the strain SR1 are transformed using standard procedures (Deblaere et al., 1987). Calli are selected on 100 ug/ml kanamycin, and resistant calli used to regenerate plants.

The techniques for transformation of Arabidopsis thaliana and Brassica napus are such that exactly the same construction, in the same vector, can be used. After mobilization to Agrobacterium tumefaciens as described hereabove, the procedures of Lloyd et al., (1986) and Klimaszewska et al. (1985) are used for transformation of Arabidopsis and Brassica respectively. In each case, as for tobacco, calli can be selected on 100 ug/ml kanamycin, and resistant calli used to regenerate plants.

In the case of all three species at an early stage of regeneration the regenerants are checked for transformation by inducing callus from leaf on media supplemented with kanamycin (see also point 6).

#### 6. Screening and analysis of transformed plants.

In the case of all three species, regenerated plants are grown to seed. Since different transformed plants can be expected to have varying levels of expression ("position effects", Jones et al., 1985), more than one transformant must initially be analyzed. This can in principle be done at either the RNA or protein level; in this case seed RNA was prepared as described (Beachy et al., 1985) and northern blots carried out using standard techniques (Thomas et al., 1980). Since in the case of both Brassica and Arabidopsis the use of the entire chimeric gene would result in cross hybridization with endogeneous genes, oligonucleotide probes complementary to the insertion within the 2S albumin were used; the same probe as used to make the construction can be used. For each species, 1 or 2 individual plants were chosen for further analysis as discussed below.

First the copy number of the chimeric gene is determined by preparing DNA from leaf tissue of the transformed plants (Dellaporta et al., 1983) and probing with the oligonucleotide used above.

The methionine content of the seeds is analyzed using known methods (Joseph and Marsden, 1986; Gehrke et al., 1985; Elkin and Griffith, 1985 (a) and (b)).

### Example II

As a second example of the method described, the same procedure is followed for the production of transgenic plant seeds with increased nutritional value by having inserted into their genome a modified 2S albumin protein from Arabidopsis thaliana having deleted its hypervariable region and replaced by way of example by a methionine rich peptide having 24 aminoacids with the following sequence :

I M M M Q P R G D M M M I M M M Q P R G M M M

All different steps going from constructs to transformants as disclosed for example I are executed with the only difference that in step 3 the following oligonucleotide has been synthesized and inserted into pBrAT2S1  
(the oligonucleotides are shown in bold type)

5' GT ATA ATG ATG ATG CAA CCA AGG GGC GAT ATG ATG ATG ATA  
ATG ATG ATG  
3' CA TAT TAC TAC TAC GTT GGT TCC CCG GTA TAC TAC TAC TAT  
TAC TAC TAC

CAA CCA AGG GGC GAT ATG ATG ATG ATA C - 3'  
GTT GGT TCC CCG CTA TAC TAC TAC TAT G - 5'

The relevant plasmids are indicated in figure 6A, details of the insertion in figure 6B and resulting aminoacid sequence of the hybrid subunit shown in figure 7. The relevant plasmids as indicated in figure 6A are pAD3, pAD7 and pTAD10.

The examples have thus given a complete illustration of how 2S albumin storage proteins can be modified to incorporate therein an insert encoding a methionine rich polypeptide followed by the transformation of plant cells such as tobacco cells, Arabidopsis cells and Brassica napus cells with an appropriate plasmid containing the corresponding modified precursor nucleic acid, the regeneration of the transformed plant cells into corresponding plants, the culture thereof up to the seed forming stage, the recovery of the seeds and finally the analysis of the methionine content of said seeds compared with the seeds of corresponding non transformed plants.

It goes without saying that the invention is not limited to the above examples. The person skilled in the art will in each case be able to choose the desired combination of appropriate aminoacids to be inserted into the hypervariable region of the 2S storage protein, in function of the plant he wants to improve with regard to its nutritional value and in function of the desired application of the modified plant.

There follows a list of bibliographic references which have been referred to in the course of the present disclosure to the extent when reference has been made to known methods for achieving some of the process steps referred to herein or to general knowledge which has been established prior to the performance of this invention. All of the said articles are incorporated herein by reference.

It is further confirmed

- that plasmid pAT2S1 has been deposited with the DSM on 4879 on October 7, 1988
- plasmids pMa5-8 has been deposited with the DSM on 4567 and pMc on 4566 on May 3, 1988
- plasmid pAT2S1Bg has been deposited with the DSM on 4878 on October 7, 1988
- plasmid pGSC1703a has been deposited with the DSM on 4880 on October 7, 1988

notwithstanding the fact that they all consist of constructs that the person skilled in the art can reproduce them from available genetic material without performing any inventive work.

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## 2S Albumin As % Of Total Seed Protein

TABLE 1

Family, species (common name)	%
<b>Compositae</b>	
<i>Helianthus annuus</i> (sunflower)	62
<b>Cruciferae</b>	
<i>Brassica</i> spp. (mustard)	62
<b>Linaceae</b>	
<i>Linum usitatissimum</i> (linseed)	42
<b>Leguminosae</b>	
<i>Lupinus polyphyllus</i> (lupin)	38
<i>Arachis hypogaea</i> (peanut)	20
<b>Lecythidaceae</b>	
<i>Bertholletia excelsa</i> (brazil nut)	30
<b>Liliaceae</b>	
<i>Yucca</i> spp. (yucca)	27
<b>Euphorbiaceae</b>	
<i>Ricinus communis</i> (castor bean)	44

C L A I M S

1. A process for producing transgenic plants with increased nutritional value which comprises :

- 5           - cultivating plants obtained from regenerated plant cells or from seeds of plants obtained from said regenerated plant cells over one or several generations, wherein the genetic patrimony or information of said plant cells, replicable within said plants, includes a nucleic acid sequence, encoding a modified 2S albumin storage protein derived from a natural 2S albumin storage protein and placed under the control of a promotor capable of directing gene expression in said plants;
- 10           . wherein said nucleic acid encodes at least part of the precursor of said 2S albumin including its signal peptide or a signal peptide of another 2S albumin, said nucleic acid being hereafter referred to as the "precursor encoding nucleic acid"
- 15           . wherein said nucleic acid contains a nucleotide sequence (hereafter termed the "relevant sequence"), which relevant sequence comprises a nonessential region of said 2S albumin modified by a heterologous nucleic acid insert or substitution for part of said nonessential region, said insert or substitution forming an open-reading phase with the non-modified parts surrounding said insert in said relevant sequence.
- 20           . wherein said insert or substitution encodes a polypeptide formed of aminoacids, identical or different from one another, with a proportion of at least one appropriate aminoacids selected from lysine, methionine, tryptophane, threonine,
- 25
- 30
- 35

phenyl-alanine, leucine, valine, isoleucine and arginine in a proportion sufficient that said modified 2S albumin storage protein is enriched in at least one of said appropriate aminoacid with respect to the contents of the same appropriate aminoacid in the natural storage protein.

2. The process of claim 1 wherein said modified 2S albumin storage protein is derived from a natural 2S storage protein which is itself foreign to the transgenic plant.

3. The process of claim 2 wherein said transgenic plant is a plant which has been transformed with a recombinant vector, e.g., a Ti-plasmid derived vector which contained said transformed storage protein.

4. The process of any of claims 1 to 3 wherein the modified storage protein is derived of a group of storage proteins obtained from the following plants:

Ricinus communis

Arabidopsis thaliana

Brassica napus

Bertholletia excelsia

5. The process of any of claims 1 to 4 wherein said modified storage protein contains a number at least of said appropriate aminoacid, e.g., lysine, methionine or tryptophane greater by at least 2, preferably by 4 than the number of the same appropriate aminoacid in the non modified storage protein.

6. The process of any of claims 1 to 5 wherein said insert is located in the region which extends in the non modified 2S albumin between the codons which code for the sixth and the seventh cysteine residues respectively, whereby the group formed of 3 codons, preferably 6 codons respectively, next to each of those which code for said sixth and seventh codons and within said region code for

the same aminoacids as corresponding groups of 3, preferably 6 codons in the non modified storage proteins.

7. The process of any of claims 1 to 6, wherein said promoter is the natural promotor of said nucleic acid.

8. The process of any of claims 1 to 6, wherein said promoter is heterologous with respect to said nucleic acid.

9. The process of any of claims 1 to 6 wherein said promotor is a constitutive promotor.

10. The process of any of claims 1 to 6 wherein said promotor is a tissue specific promotor.

11. The promotor of claim 10 which is a seed specific promotor.

12. The process of any of claims 1 to 11, wherein the heterologous insert is foreign to the natural nucleic acid encoding the precursor of said 2S albumin.

13. The process of any one of claims 1 to 12, wherein the heterologous insert contains a segment as above-defined normally present in the genetic patrimony or information of said seeds or plant cells, the "heterologous" character of said insert then addressing to the one or several codons which surround it, on both sides thereof and which link said segment to the non modified parts of the nucleic acid encoding said precursor.

14. A recombinant DNA which includes a nucleic acid sequence, which can be transcribed into the mRNA encoding at least part of the precursor of a 2S albumin including the signal peptide of said plant, said nucleic acid being hereafter referred to as the "precursor encoding nucleic acid" :

. wherein said nucleic acid contains a nucleotide sequence (hereafter termed the "relevant sequence"), which relevant sequence comprises a

nonessential region modified by a heterologous nucleic acid insert forming an open reading frame in reading phase with the non modified parts surrounding said insert in said relevant sequence.

5       . wherein said insert includes a nucleotide segment encoding a polypeptide containing said, appropriate aminoacids as defined in any of claims 1 to 12.

10       . wherein said precursor coding nucleic acid is placed under the control of a promoter capable of directing gene expression in plants.

15       15. The recombinant DNA of claim 14 which is a plasmid.

16. The recombinant DNA of claim 15 which is capable of transforming plant cells and of causing the replication of said modified precursor nucleic acid sequence in said plant cells.

20       17. The recombinant DNA of claim 16 which is a Ti-derived plasmid.

25       18. As a regenerable source of appropriate aminoacids with high nutritional value, which is formed of either plant cells of a seed-forming plant, which plant cells are capable of being regenerated into the fullplant or seeds of said seed-forming plants wherein said plants or seeds have been obtained as a result of one or several generations of the plants resulting from the regeneration of said plant cells, wherein further the DNA supporting the genetic information of said plant cells or seeds  
30       comprises a nucleic acid or part thereof, including the sequences encoding the signal peptide, which can be transcribed in the mRNA corresponding to the precursor of a 2S albumin in said plant, placed under the control of a promoter capable of directing gene expression in plants,  
35       and

- 5                   . wherein said nucleic acid sequence contains a relevant modified sequence encoding the mature 2S albumin or one of the several sub-sequences encoding for the corresponding one or several sub units of said mature storage protein,
- 10                   . wherein further the modification of said relevant sequence takes place in one of its non essential regions and consists of a heterologous nucleic acid insert forming an open-reading frame in reading phase with non modified parts which surround said insert in the relevant sequence,
- . wherein said insert or substitution is as defined in any of claims 1 to 12.

15                   19. The source of polypeptide of claim 18, wherein said insert is in a synthetic man-made oligonucleotide.

                  20. The source of polypeptide of claim 18, wherein said insert is obtained from a prokaryotic or eukaryotic organism.

20                   21. The source of polypeptide of claim 18, 19 or 14, wherein the heterologous segment contained in said insert encodes a non plant variety specific polypeptide.

25

30

35

# FIGURE 1

## ORGANIZATION OF 2S ALBUMIN PRECURSORS

Comparison of protein and nucleic acid sequences reveals that the preproprotein is processed at five points.

Signal peptide    A.T.P.F.    Small Subunit I.P.F.    Large Subunit    C.T.P.F.

1/21

Arabidopsis

Brassica

Bertholletia

21	16	36	10	79	2
21	16	29,35	19	86	1
22	14	34	5	73	4

# Fig. 2

## COMPARISON OF 2S ALBUMIN PRECURSOR PROTEIN SEQUENCES

### Signal Peptide

B. exce.  
B. napus  
AT2S1  
AT2S2  
AT2S3  
AT2S4

M	A	K	I	S	V	A	A	A	A	L	L	V	L	M	A	L	G	H	A	T	A
M	A	N	K	L	F	L	V	S	A	T	L	A	F	-	F	F	L	L	T	N	A
M	A	N	K	L	F	L	V	C	A	A	L	A	L	-	C	F	L	L	T	N	A
M	A	N	K	L	F	L	V	C	A	T	F	A	L	-	C	F	L	L	T	N	A
M	A	N	K	L	F	L	V	C	A	T	L	A	L	-	C	F	L	L	T	N	A
M	A	N	K	L	F	L	V	C	A	A	L	A	L	-	C	F	I	L	T	N	A

(22)  
(21)  
(21)  
(++)  
(++)  
(++)

### Amino Terminal Processed Fragment

B. exce.  
B. Napus  
AT2S1  
AT2S2  
AT2S3  
AT2S4

-	F	R	A	T	V	T	T	T	V	V	-	E	E	E	N
S	I	Y	R	T	V	V	E	F	D	E	D	D	A	T	N
S	I	Y	R	T	V	V	E	F	E	E	D	D	A	T	N
S	I	Y	R	T	V	V	E	F	D	E	D	D	A	S	N
S	I	Y	R	T	V	V	E	F	E	E	D	D	A	S	N
S	V	Y	R	T	V	V	E	F	D	E	D	D	A	S	N

(14)  
(16)  
(16)  
(+)  
(++)  
(++)

• • • • •

— — — — —

R. comm.	Y	I	-	K	Q	Q	Q	V	-	S	G	Q	G	P	R	R	(34)
B. exce.	Y	M	R	Q	Q	M	A	E	S	S							(28)
B. napus	W	L	H	K	Q	Q	A	M	Q	S	G						(35, 29)
AT2S1	L	M	L	Q	Q	A	R	Q	Q	-	G	R	S	D			(36)
AT2S2	L	M	R	M	Q	M	R	Q	Q	-	G	R	G	G			(++)
AT2S3	W	M	S	K	Q	M	R	Q	Q	-	G	R	G	G			(++)
AT2S4	W	M	R	K	Q	M	W	Q	Q	-	G	R	G	G			(++)

## Fig. 2 (cont.2)

# Internal Processed Fragment

	B.	excel.	P	Y	Q	T	M
B. napus	G	-	G	P	-	T	L D G E F D F E D D M E N
AT2S1	-	-	-	-	-	-	- - - - E F D D M E N
AT2S2	-	-	G	P	-	S	L D D E F D L E D D I E N
AT2S3	-	-	G	P	-	S	L D D E F D M E D D I E N
AT2S4	-	-	G	P	-	S	L D D E F D M E D D I E N

## Large Subunit

R. comm.	Q	E	R	-	S	L	R	G	C	C	C	D	H	L	K	Q	M	Q	-	S	Q	-
B. excel.	P	R	R	G	M	E	P	H	P	Q	Q	E	Q	L	E	G	M	D	-	S	P	-
B. napus	P	Q	G	P	Q	Q	R	P	Q	L	Q	N	E	L	H	Q	-	-	E	E	P	P
AT2S1	P	Q	G	P	Q	Q	E	Q	Q	L	Q	N	E	L	R	Q	-	-	E	E	P	P
AT2S2	P	Q	G	P	Q	Q	G	H	Q	L	Q	N	E	L	R	Q	-	-	E	E	P	P
AT2S3	F	E	G	P	Q	Q	G	Y	Q	L	Q	N	E	L	R	Q	-	-	E	E	P	P
AT2S4	P	-	-	-	-	-	Q	R	R	Q	Q	C	C	L	L	Q	-	-	E	E	P	P

R.	comm.
B.	excel.
B.	napus
AT2S1	
AT2S2	
AT2S3	
AT2S4	

R.	comm.
B.	excel.
B.	napus
AT2S1	
AT2S2	
AT2S3	
AT2S4	

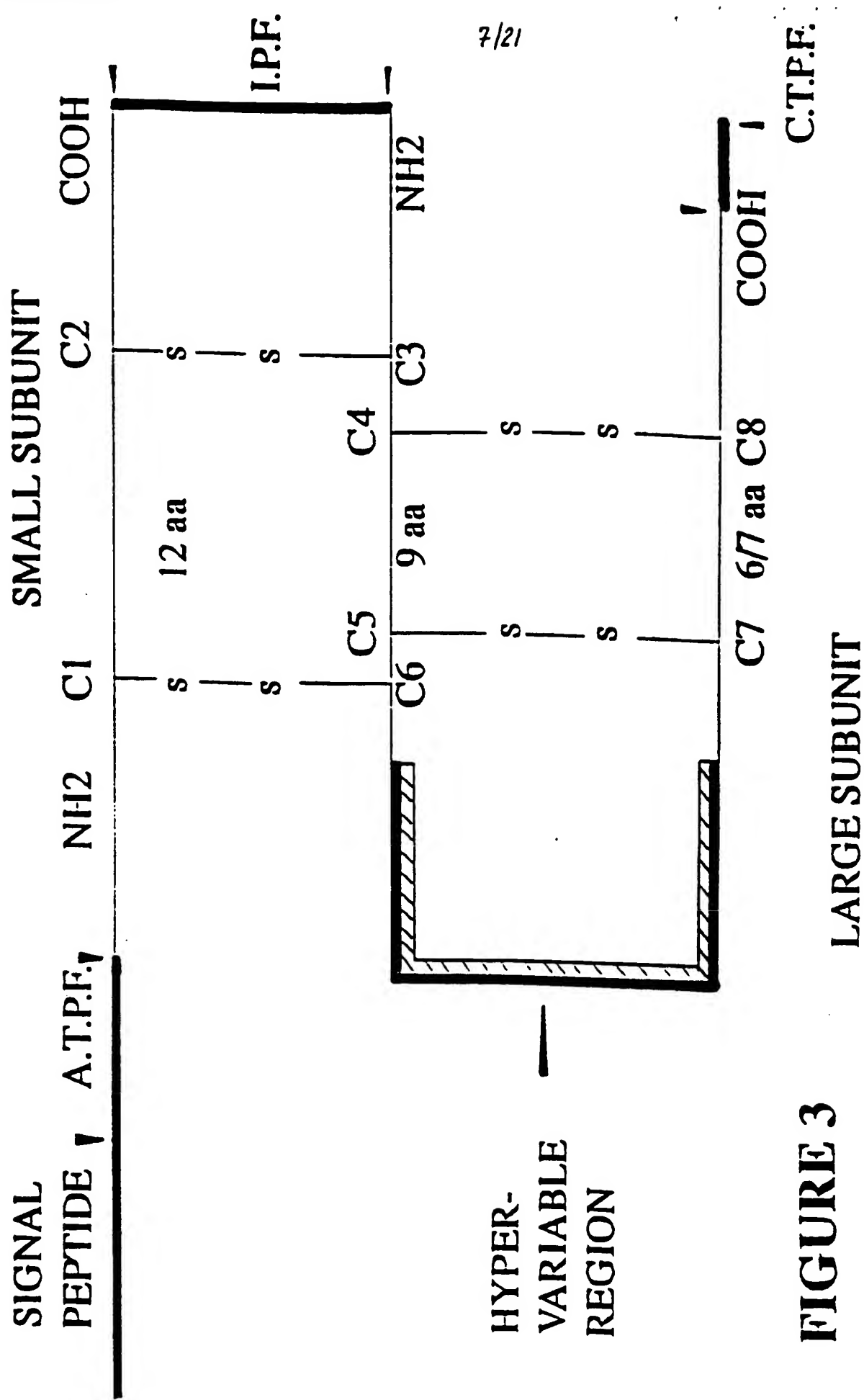
ALZCO  
AMCOA

ATZS3

R. comm.	Q	-	-	C	R	F						(61)				
B. excel.	R	-	-	C	P	M	G	G	S			(73)				
B. napus	V	S	V	C	P	F	Q	K	T	M	P	G	-	P	S	(86)
AT2S1	V	D	V	C	P	F	N	-	I	P	S	F	P	S	(79)	
AT2S2	V	G	E	C	P	F	Q	T	T	I	P	F	P	P	(++)	
AT2S3	V	G	E	C	P	F	Q	T	T	I	P	F	P	P	(++)	
AT2S4	V	G	V	C	P	F	Q	-	-	I	P	S	I	P	S	(++)

**Carboxyl Terminal Processed Fragment**

	I	A	G	F	
B. excel.	Y	-			( 4)
B. napus	F	Y			( 1)
AT2S1					( 2)
AT2S2	Y	-			( ++)
AT2S3	Y	Y	Y		( ++)
AT2S4	Y	Y	Y		( ++)



**FIGURE 3**

Figure 4.

ATCTTTATCCA -421  
 TATAATTGCTTACCATCAATAGACAATATCCAATGACCGGTGACCTGGGTGATTAAGTA -361  
 ATTTTCAAGATGCTAAACTTTTATGTATTTCAGAAATTAACCTCCAAACATTATTG -301  
 ACACACTACTACTCTTTCCGTATTGACTCTCACTCACTAGTCATTTCAAATAATTGACATGT -241  
 CAGAACATGAGTTACATCGTTGCCATATTGCCAAGTAGACGGGAACTTGTCACTTCCT -181  
 TTACATTGAGTTTCCACACCTAATCAGGACAACAATCATATAGCTCTCGCATACAAC -121  
 AAACATATGCATGTATTCTTACAGTGAACCTCCATGCCAGTCTCTTTTCTCACCTATAAA -61  
 TACCAACACACCTTCACCACATTCTTCACTCGAACCAAAACATACACACATAGCAAAAA -1

M A N K L F L V C A A L A L C F L L T N 20  
 ATGGCAACAAGTTGTTCCCTCGTCTGGCAGCTCTCGCTCTCTGCTTCTCTCTCACCAC 60

\*Start SSU

A S I Y R T V V E F E E D D A T N P I G 40  
 GCTTCCATCTACCGACCGTCTGTGAGTTCGAGAGATGACGCCACTAACCCCATAGGC 120

Fig. 4 (cont. 1)

P K M R K C R K E F Q K E Q H L R A C Q 60  
 CCAAATGAGCAATCCCGCAAGGAGTTTCAGAAAGAACACACCTAAGAGCTTGCCAG 180

\*Processed -->

Q L M L Q Q A R Q Q G R S D \*E F D F E D D 80  
 CAATTGATGCTCCAGCAAGCAAGCGGAGCCGCTAGCGATGAGTTTGATTTTCGAAGCGAC 240

\* Large subunit -->

M E N \*P Q G Q Q E Q Q L F Q Q C C N E 100  
 ATGGAGAACCCACAGGGACACAGCAGGAAACACAGCTATTCCAGCAGTGCTGCAACGAG 300

L R Q E E P D C V C P T L K Q A A K A V 120  
 CTTGCCAGGAGAGCCAGATTGCTGTTGGCCCCACCTTGAAACAAGCTGCCCAAGCCGTT 360

oligonucleotide 5'-CAAGCTGCCAAGTACGGT

K Y G

Fig. 4 (cont. 2)

R L Q G Q H Q P M Q V R K I Y Q T A K H 140  
AGACTCCAGGACAGACCAACCAATGCAAGTCAGGAAATTACAGACAGCCAAAGCAC 420  
GGATTCTTGAAGCAGCAAC-3' oligo

G F L K

L P N V C D I P Q V D V C P F N I P S F 160  
TTGCCCAACGTTTGGACATCCCGCAAGTTGATGTTTGTCCCTTCAACATCCCTTCATTC 480

\*End mat. lg. su.

P S\* F Y \* 164

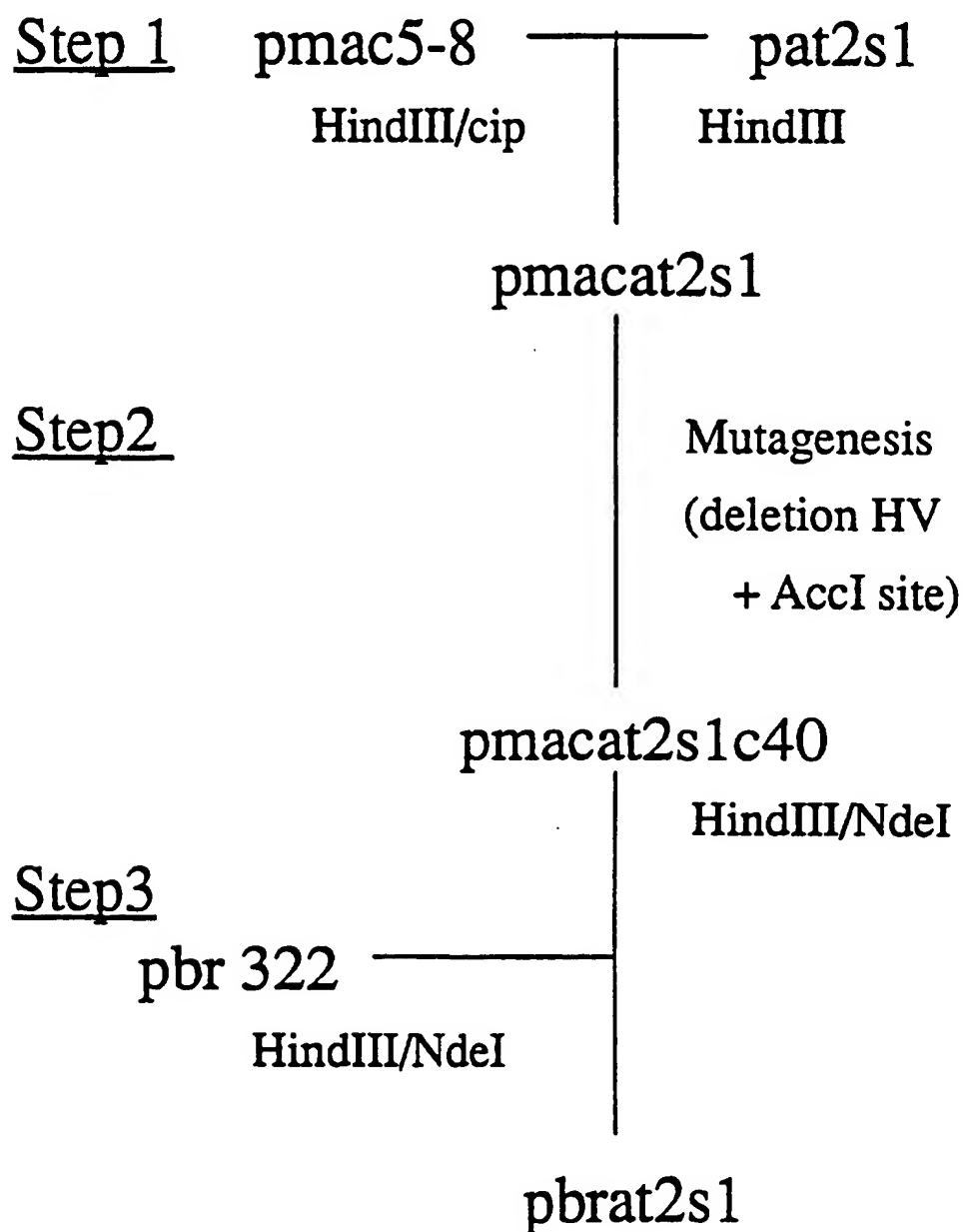
CCTTCTTCTACTAATCTCAACAACCCCTCAAGCGTATGAGAGTGTGGTTGTGATA 540

TATACATGTTGACACTTGACACATACCACACCTCATCGTGTGTTTATGATAAATGT 597

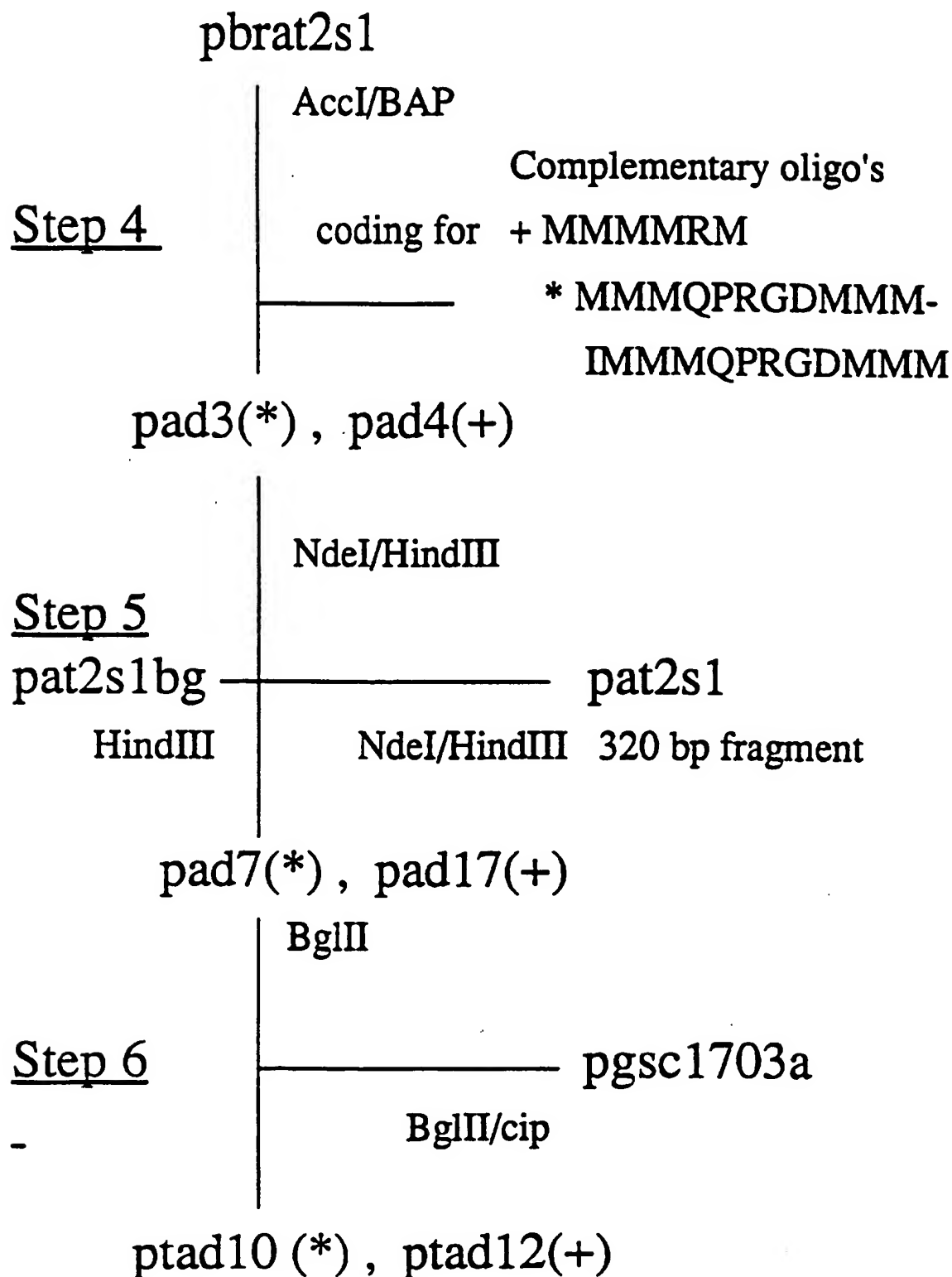
Figure 5

GGGCAICAGCAICAGCAITTTTICAGCAITGTGTGAIGA	
P Q G Q Q E Q Q L F Q Q C C N E L R Q E E P D C V C P T L	
AAICAGCIGCIAAGCIGTIGTITTCAGGICAGCAICA	CCIAAI
K Q A A K A V R L Q G Q H Q P M Q V R K I Y Q T A K H L P N	
GTITGICAGATCCICAGTIGAGTITGICCTTTAAICC	
V C D I P Q V D V C P F N P	

**Fig. 6A:** flowchart of constructions showing successive steps in the deletion of sequences encoding most of the hypervariable region of the Arabidopsis 2S and their replacement with sequences rich in methionine codons



# Fig. 6A (cont.)



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R. comm.																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																				</
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Fig. 6B (cont. 1)

R. comm.	G	-	-	Q	N	V	F	E	A	F	R	T	A	A	N	L	P	S	M	7	C	G	V	S	P	T
B. excel.	G	E	-	Q	M	-	R	R	M	R	L	A	E	N	I	P	S	R	C		N	L	S	P	M	
B. napus	G	K	Q	Q	M	V	S	R	I	Y	Q	T	A	H	I	P	K	V	C		N	I	-	P	Q	
AT2S1	-	-	-	-	-	V	R	K	I	Y	Q	T	A	K	H	L	P	N	V		C	D	I	-	P	Q
AT2S2	-	-	-	-	-	S	R	K	I	Y	K	T	A	K	Y	L	P	N	I		C	K	I	-	Q	Q
AT2S3	-	-	-	-	-	S	R	K	I	Y	Q	S	A	K	Y	L	P	N	I	C	K	I	-	Q	Q	
AT2S4	-	-	-	-	-	V	R	K	I	Y	Q	A	A	K	Y	L	P	N	I	C	K	I	-	Q	Q	

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R. comm.	Q	-	-	R	F	8	C	R	F	M	G	G	S
B. excel.	R	-	-	C	P		C	P	M	G	G	S	
B. napus	V	S	V	C	P		F	Q	T	-	-	-	
AT2S1	V	D	V	C	P		F	Q	N	-	-	-	
AT2S2	V	G	E	C	P		F	Q	T	-	-	-	
AT2S3	V	G	E	C	P	F	Q	T	-	-	-		
AT2S4	V	G	V	C	P	F	Q	T	-	-	-		

# Fig. 6B (cont. 2)

Amino acid sequence between the 5th and 7th cysteines resulting from the deletion of the HV region of AT2S1 and insertion of an AccI site

<sup>5</sup>  
<sup>6</sup>  
<sup>7</sup>  
V P T L K G I H L P N V D I

Details of the insertions into the AccI site made in examples 1 and 2. The bases originating from the AccI site are shown in bold print.

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GT' AT AC  
CA TA' TG      AccI site

AAA GGT ATA ATG ATG ATG ATG CGC ATG **ATA** CAC  
TTT **CCA** **TAT** TAC TAC TAC TAC GCG TAC **TAT** GTG      Example I

AAA CGT ATA ATG ATG ATG CAA CCA AGG GGC GAT ATG ATG ATA ATG ATG A  
TTT GCA **TAT** TAC TAC TAC GTT GGT TGG GCG GTA TAC TAC TAT TAC TAC T  
CAA CCA AGG GGC GAT ATG ATG **ATA** CAC  
GTT GGT TCC GCG CTA TAC TAC TAT GTG      Example II

**Figure 7** Amino acid sequences of the large subunits of the Arabidopsis 2S protein, that remaining after the deletion of most of the hypervariable region, and of the two modified Arabidopsis 2S proteins.

AT2S1 = unmodified large subunit of AT2S1  
 Del HV = deletion of the AT2S1 HV region  
 Subst 1 = substitution by IMMMMRM  
 Subst 2 = substitution by

IMMMQPRGDMMMIMMMQPRGDMMM

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AT2S1	P	Q	G	Q	Q	Q	E	Q	Q	L	F	Q	Q	C	C	N	E	L	R	Q	-	-	E	E	P	D	C	V	C	P	T	L	K	-	-	
Del HV	P	Q	G	Q	Q	Q	E	Q	Q	L	F	Q	Q	C	C	N	E	L	R	Q	-	-	E	E	P	D	C	V	C	P	T	L	K	G	-	
Subst 1	P	Q	G	Q	Q	Q	E	Q	Q	L	F	Q	Q	C	C	N	E	L	R	Q	-	-	E	E	P	D	C	V	C	P	T	L	K	G	I	
Subst 2	P	Q	G	Q	Q	Q	E	Q	Q	L	F	Q	Q	C	C	N	E	L	R	Q	-	-	E	E	P	D	C	V	C	P	T	L	K	G	I	
AT2S1	Q	A	A	K	A	V	R	L	Q	G	Q	H	Q	P	M	Q	-	V	R	K	I	Y	Q	T	A	K	-	H	L	P	N	V	C	D	I	
Del HV	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	H	L	P	N	V	C	D	I	
Subst 1	M	M	M	R	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	H	L	P	N	V	C	D	I	
Subst 2	M	M	M	Q	P	R	G	D	M	M	M	I	M	M	M	Q	P	R	G	D	M	M	M	-	-	-	-	I	H	L	P	N	V	C	D	I

AT2S1	-	P	Q	V	D	V	C	P	F	N	-	-	I	P	S	F	P	S
Del HV	-	P	Q	V	D	V	C	P	F	N	-	-	I	P	S	F	P	S
Subst 1	-	P	Q	V	D	V	C	P	F	N	-	-	I	P	S	F	P	S
Subst 2	-	P	Q	V	D	V	C	P	F	N	-	-	I	P	S	F	P	S

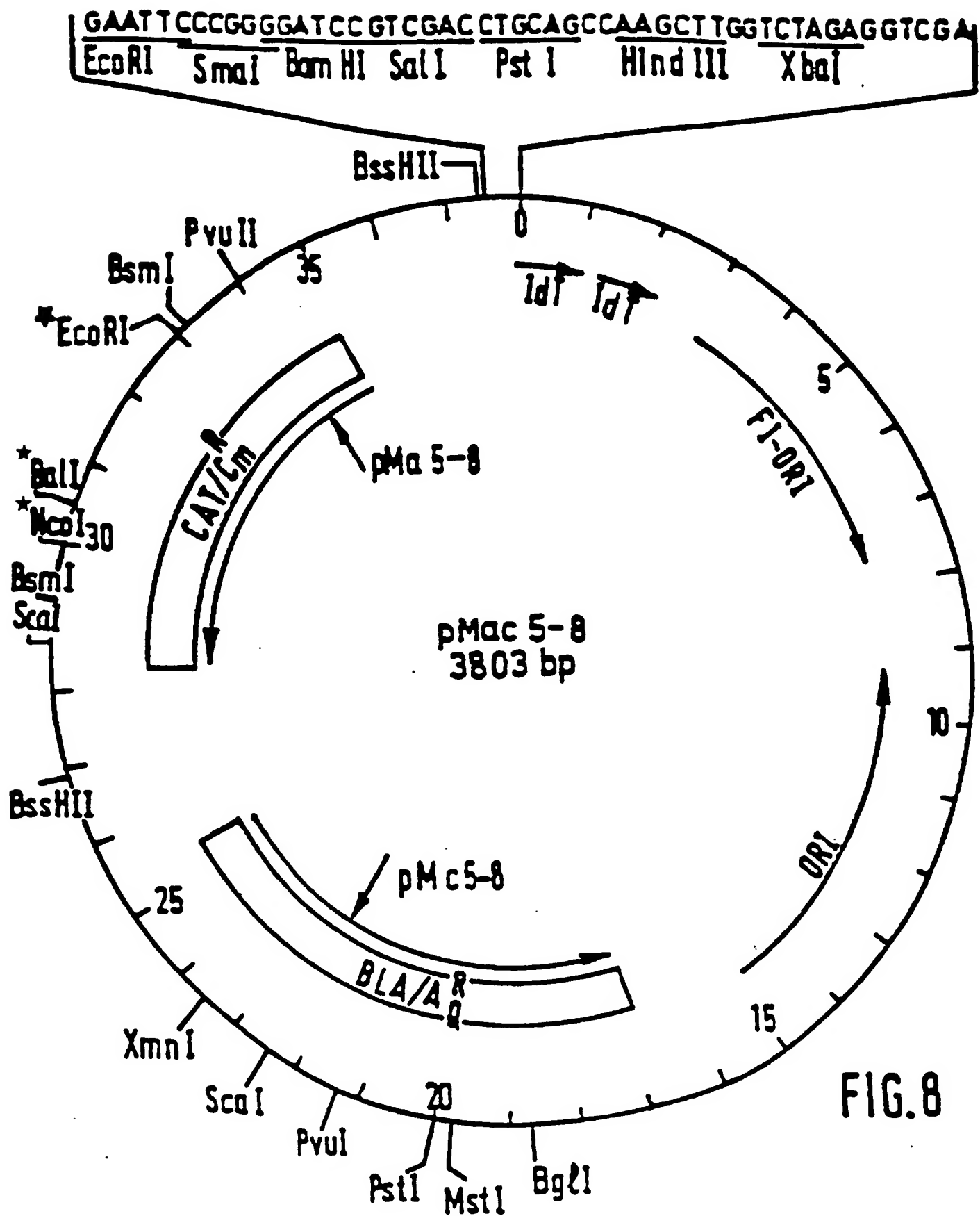
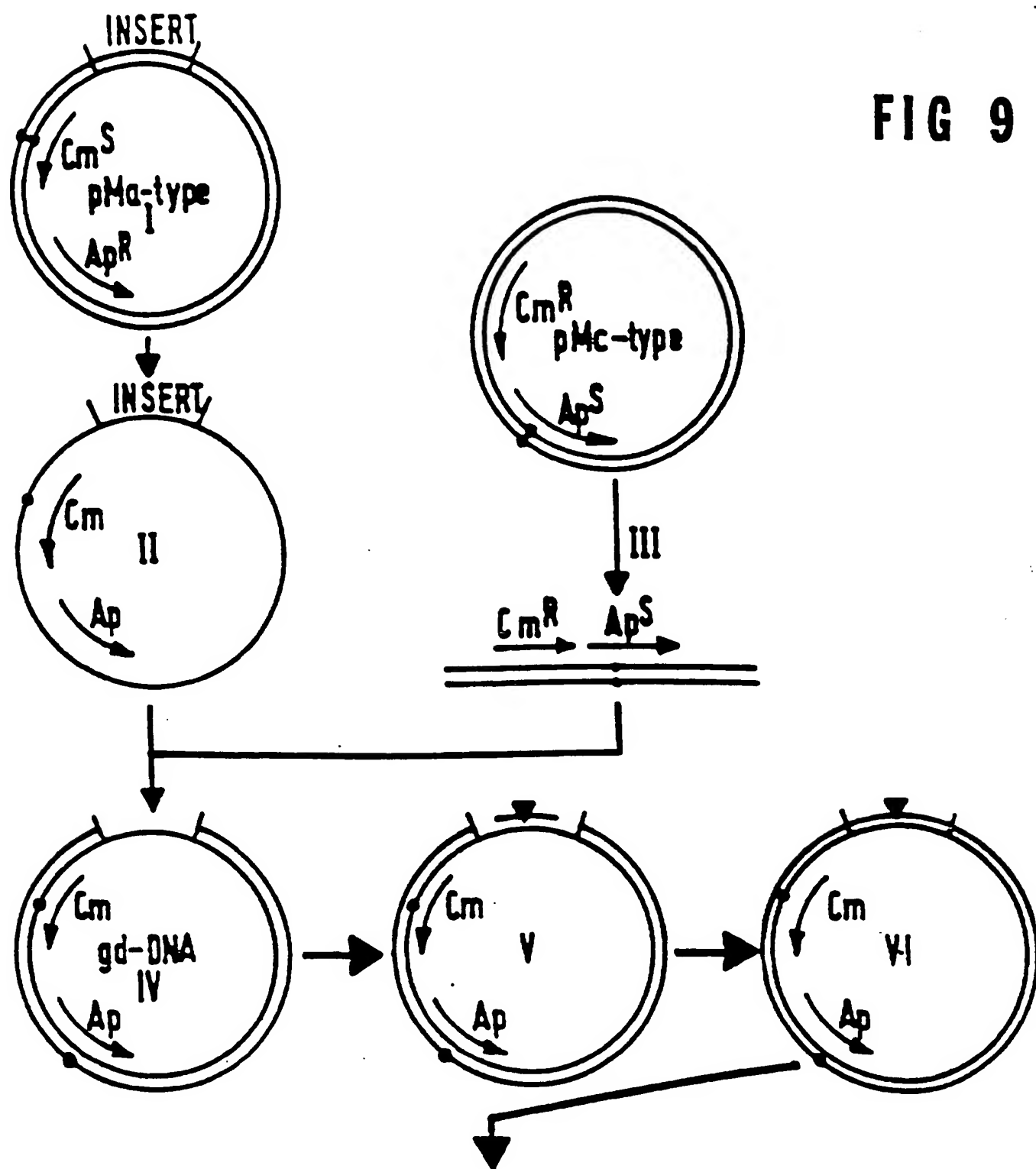
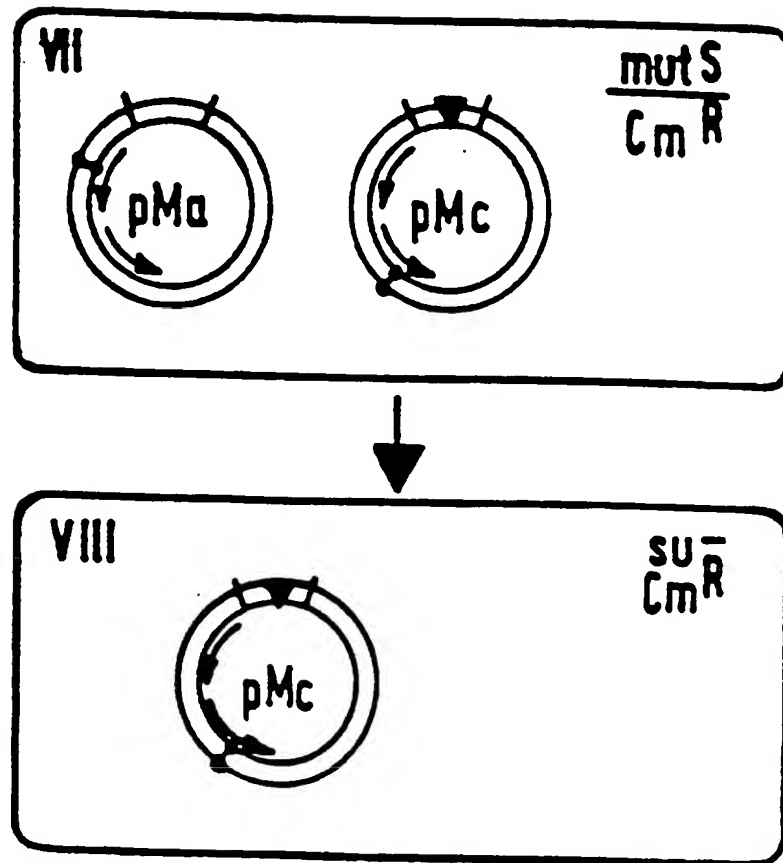
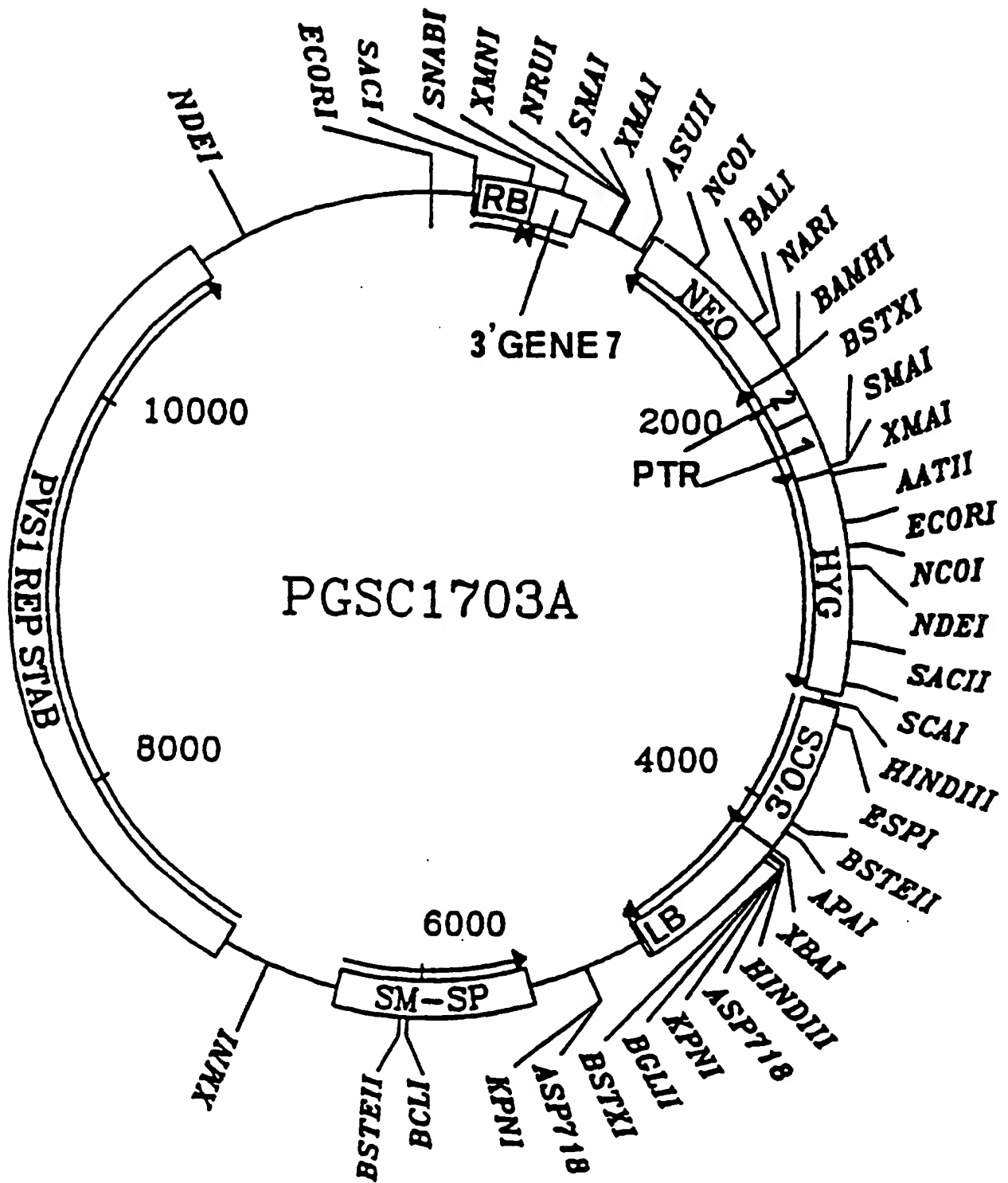


FIG 9

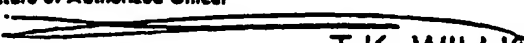


**FIG 9 (continued)**

**FIG 10**

# INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 89/01229

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC <sup>5</sup> : C 12 N 15/82, C 12 N 15/29, C 12 N 5/14, A 01 H 5/00		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched *		
Classification System *	Classification Symbols	
IPC <sup>5</sup>	C 12 N, A 01 H	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT *</b>		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. **
X	WO, A, 87/07299 (CALGENE) 3 December 1987 see page 25, line 24 - page 28, line 10	1-5,7, 12,13-21
Y	--	8-11
X	Theoretical and Applied Genetics, volume 75, no. 5, 1988, Springer- Verlag, S.E. Radke et al.: "Transformation of Brassica napus L. using Agrobacterium tumefaciens: developmentally regulated expression of a reintroduced napin gene", pages 685-694 see the whole article	1-5,7, 12,13-21
Y	--	8-11
P,X	WO, A, 89/03887 (PLANT GENETIC SYSTEMS) 5 May 1989 see the whole document --	1-4,6-21
./.		
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: **</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
6th February 1990	22. 03. 90	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	 T.K. WILLIS	

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**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

EP 8901229

SA 32070

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EP-A- 0318341	31-05-89	AU-A- 2811889 WO-A- 8903887 EP-A- 0319353	23-05-89 05-05-89 07-06-89
DD-A- 240911		None	